10 / 597636

Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/BE05/000017

International filing date: 07 February 2005 (07.02.2005)

Document type: Certified copy of priority document

Document details: Country/Office: EP
Number: 04447027.6
Filing date: 06 February 2004 (06.02.2004)

Date of receipt at the International Bureau: 25 April 2005 (25.04.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

World Intellectual Property Organization (WIPO) - Geneva, Switzerland
Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse

PCT/BE 2005/000017

BE 05/17

Europäisches Patentamt

European Patent Office

Office européen des brevets

REÇU
25.-3-2005
OFFICE VAN MALDEREN

Bescheinigung

Certificate

Attestation

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. Patent application No. Demande de brevet n°

04447027.6

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

R C van Dijk

Anmeldung Nr:
Application no.: 04447027.6
Demande no:

Anmeldetag:
Date of filing: 06.02.04
Date de dépôt:

Anmelder/Applicant(s)/Demandeur(s):

Université de Liège
25, quai Van Beneden
4020 Liège
BELGIQUE

Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se referer à la description.)

Method and kit for measurement of neutrophil cell activation

In Anspruch genommene Prioriät(en) / Priority(ies) claimed /Priorité(s) revendiquée(s)
Staat/Tag/Aktenzeichen/State/Date/File no./Pays/Date/Numéro de dépôt:

Internationale Patentklassifikation/International Patent Classification/
Classification internationale des brevets:

G01N33/48

Am Anmeldetag benannte Vertragstaaten/Contracting states designated at date of filing/Etats contractants désignées lors du dépôt:

AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IT LU MC NL
PT RO SE SI SK TR LI

METHOD AND KIT FOR THE MEASUREMENT OF NEUTROPHIL CELL ACTIVATION

Field of the invention

[0001] The present invention is related to methods and kits for the measurement of equine myeloperoxidase (MPO), a specific enzyme of equine neutrophils, either *in toto* [first method], or specifically in its active form [second method]. Said methods and kits, used independently or in combination, find improved applications in the veterinary field and can be adapted for application in human health care. The concept of the second method is applicable to any other enzyme.

Background of the invention

[0002] Myeloperoxidase (MPO) is a specific enzyme of polymorphonuclear leucocytes (also known as neutrophils), which are white blood cells specialized in the fight against micro-organisms by phagocytosis.

[0003] Pathogens are destroyed inside the neutrophils by proteinases and myeloperoxidase, this latter enzyme being specifically responsible for the production of a potent oxidant agent, hypochlorous acid (HOCl). This HOCl (bleach) allows the destruction of the bacterial polysaccharidic capsules that withstand proteinases. MPO thus plays a key role in the host defense against infection.

[0004] During their fight against micro-organisms, dying neutrophils release myeloperoxidase in the

surrounding liquids and tissues. When the activation of the neutrophils is excessive and becomes uncontrolled (as in acute inflammation pathologies), the release of myeloperoxidase is important and high concentrations of this enzyme are reached in biological media or samples (plasma, tissues, ascite fluids, broncho-alveolar fluids, pleural fluid, lymph, urine, saliva, uterine irrigation liquids...), leading to an increased risk of cytotoxicity (by myeloperoxidase capture into cells or binding on cell surface, with *in situ* production of oxidants).

[0005] Until now, in equine medicine, myeloperoxidase was measured by the detection of a peroxidase activity. However, said detection according to hitherto developed techniques is not specific for equine myeloperoxidase (a general peroxidase activity is detected), is tedious and not applicable to complex biological media and samples (such as plasma) due to the presence of proteins (albumin, lipoproteins, ...) and reducing agents that interfere with the enzymatic measurement.

[0006] No efficient technique has been developed hitherto for the measurement of the total concentration of equine myeloperoxidase in complex biological fluids (both cellular and acellular) and in tissues.

[0007] The presence of myeloperoxidase in alveoli and tissues is presently estimated by the measurement of a non-specific peroxidase activity after extraction.

[0008] There is an ever increasing demand for easy and improved diagnostic tests to measure MPO activity and concentrations, especially in horses. The design of a rapid and sound assay for equine myeloperoxidase measurement is highly wanted for the diagnosis of equine diseases and/or pathologies (such as for instance colics with a high mortality in *Equidae*, sepsis, acute lung injury, acute

inflammation, ...). The choice between a clinical treatment, a surgical intervention (costly for the veterinary surgeon and for the breeder) or, at the worst, euthanasia of the animal will be easier to make and the decision taken will be better funded when good, rapid and reliable assays exist to diagnose excessive neutrophil activation and/or invasion.

[0009] Myeoloperoxidase is known to be a specific marker for excessive neutrophil activation and/or invasion, in humans as well as other mammals such as horses. In horses, intestinal tissue scores correlated for instance positively with tissue MPO activity in adjacent specimens (Mc Connino et al., 1999, Am J Vet Res **60**: 807-813). It has been established that the physiological values of plasma myeloperoxidase in healthy horses are significantly exceeded in several acute abdominal pathologies, in horses with large intestine strangulation and in horses which will not survive (Deby-Dupont et al, 1998, Vet Immunol Immunopathol. **66**: 257-271; Grülke et al., 1999, Can J Vet Res. 63:142-7; Grülke, 2002, doctoral thesis, ISBN 2-930212-57-8). In humans, the plasma concentration of myeloperoxidase is currently taken as marker of neutrophil activation such as in cardiovascular diseases (Zhang et al., 2001, JAMA ***286***: 3126-2142).

State of the art

[0010] The publication of Deby-Dupont et al. (1998, Vet Immunol Immunopathol. **66**: 257-271) describes the preparation of a radioimmunological assay (RIA) which requires the use of radioactively labelled molecules, specialized equipment and specific authorization for the use of said radioactive isotope labels.

[0011] Said radioimmunological assay (RIA) is suitable for equine myeloperoxidase detection *in toto*

(without distinction between the active or non active forms of the enzyme, recognizing for instance also hemi-enzymes and the heavy subunits of MPO). The available RIA method is, however, not suitable for the targeted detection of enzymatically active myeloperoxidase. The RIA method can be used for myeloperoxidase detection in plasma, but is not suited for adequate and reliable detection of MPO in tissue samples and in complex biological media or samples (such as seminal plasma, broncho-alveolar lavage fluids, sputum, purulent liquids, abscess, pleural fluids, urine, saliva, uterine irrigation liquids, ...). An adequate and reliable measurement of MPO in complex media and samples is not possible, due to interferences of the medium leading to a proteolytic alteration of the labelled reference molecule (^{125}I-labelled myeloperoxidase), and due to the high viscosity, excessive lipid and low protein contents of the medium altering the double antibody complex formation and precipitation ("matrix effects").

[0012] The international patent application WO 99/61907 describes a method for measuring the activation status of leucocyte cells, which cannot distinguish between lymphocytes (T-lymphocytes, NK or B-lymphocytes), eosinophils, neutrophils, basophils, monocytes and macrophages. Said method further requires the presence of said cells (*in casu* the leucocyte cells) in the biological sample to be analyzed. The activation status of said leucocyte sub-population is obtained by the measurement of the size of the leucocytes and/or by the measurement of the peroxidase activity of said leucocyte cells. Among the total peroxidase activities detected are, at least, the peroxidase activities of eosinophils (due to eosinophil peroxidase: EPO) and of neutrophils (due to the myeloperoxidase: MPO).

[0013] The method described in WO 99/61907 thus detects all kinds of peroxidase activity, is not limited to myeloperoxidase activity per se, and measures peroxidase activity in general in neutrophils, eosinophils and other blood cell types. The method is merely confined to the measurement of peroxidase activity in a sample of isolated cells, wherein peroxidase activity is anyhow high due to the *in situ* release of enzymes by the cells.

[0014] Only active intracellular enzyme activity is measured in the method according to WO 99/61907, for instance via a flow cytometer or an automated haematology analyzer, which require the availability of highly skilled personnel.

[0015] The method of WO 99/61907 does not apply specifically to the measurement of myeloperoxidase from neutrophils and does not apply to complex acellular media such as plasma and to tissues. Therefore, this method is not specific enough for myeloperoxidase and will not allow the practitioner to identify clearly the presence/absence of a given disease, or the condition of a specific disease, which is characterized by a specific activation status of neutrophil cells.

[0016] Finally, depending on the oxydo-reduction status of the sample milieu, major artefacts could arise and affect the precision of detection when the person skilled in the art will apply the method described in the document WO 99/61907.

[0017] The document WO 02/50550 describes the use of recombinant human myeloperoxidase for obtaining oxidized lipoprotein and discloses corresponding monoclonal antibodies directed against them. Said antibodies are suitable for diagnostic, preventive and therapeutic uses, especially for diagnosing and determining cardiovascular

risks linked to the presence of oxidized low density lipoproteins.

Aims of the present invention

[0018] The present invention aims to provide new methods and kits for a specific measurement of neutrophil cell activation in a biological sample obtained from a mammal, preferably a horse.

[0019] A main aim of the present invention is to provide such methods and kits which are specific for the measurement of myeloperoxidase obtained from mammalian, preferably equine neutrophils, in complex cellular or acellular biological media.

[0020] A further aim of the present invention is to provide methods and kits which can characterize total (active and non-active) myeloperoxidase [first method], and to provide methods and kits which can characterize exclusively active myeloperoxidase obtained from said neutrophil cells [second method].

[0021] A last aim of the present invention is to provide improved methods and kits for veterinary and medical applications.

Summary of the invention

[0022] The present invention is related to an *in vitro* method to measure the activation status of neutrophil cells present in a biological sample obtained from a mammal, which method specifically measures the myeloperoxidase (MPO) content only, said content being correlated with said neutrophil activation status, said method comprising the steps of:

- obtaining a biological sample, preferably a biological fluid from said mammal, said sample

containing said cells or containing MPO released by said cells,

- capturing MPO that is present in said biological sample by specific antibodies,
- detecting and/or measuring either total (active and inactive) MPO or exclusively active MPO present in said biological sample,
- possibly comparing the measured MPO values with normal MPO levels obtained from a significant number of healthy mammals or optionally quantifying MPO levels using a standard MPO curve, and
- relating the normalized MPO levels measured to an activity status of said neutrophils indicative of the presence, absence or condition of a disease or immunological status;

said detection and/or measurements specifically and accurately representing total MPO levels or active MPO levels in any type of biological sample.

[0023] To measure the total MPO content, a method further referred to as a MYELO-ELISA was developed. To measure active MPO enzyme levels, a method further referred to as a MYELO-SIEFED was developed.

[0024] In a preferred embodiment said mammal is a horse and said myeoloperoxidase (MPO) an equine myeoloperoxidase.

[0025] Preferably, MPO standard curves are established for the specific detection method used and, if possible, for the type of sample analyzed.

[0026] Advantageously, measured MPO values are normalized in view of mean MPO levels obtained from a significant number of healthy individuals or mammals, preferably horses. This can be of particular importance as it was observed that the response of neutrophils can be

highly variable from one individual to another but also from one day to another for one and the same individual.

[0027] Advantageously, normalized MPO levels can be linked to the absence or presence of a disease and/or pathology or can be linked to a specific condition or status therein, by comparing measured levels with cut-off values derived from measurements performed on a significant number of individuals with said disease and/or pathology and/or in a specific condition and/or status. As such, the methods according to the present invention can also be used to make predictions on the susceptibility of individuals and/or groups for certain diseases and/or pathologies. Such prediction could be highly useful in veterinary fields such as horse breeding.

[0028] Advantageously, following the methods according to the invention, the neutrophil activation status is detected and/or measured via an immunological reaction wherein MPO is specifically captured via MPO-recognizing antibodies and then detected via a colorimetric reaction, either directly (detection of MPO activity; MYELO-SIEFED) or indirectly ("immunological sandwich" with a second MPO antibody and an enzyme-bearing antibody, followed by the detection of this enzyme activity; MYELO-ELISA).

[0029] Said MPO-recognizing or MPO-specific antibodies can be polyclonal or monoclonal antibodies, fragments thereof, engineered antibodies such as humanized antibodies (all obtainable by techniques well known in the art) as long as they are specific in their recognition of MPO in a complex medium possibly containing other types of peroxidases.

[0030] An aspect of the invention relates to a monoclonal antibody raised against equine MPO, which was not available hitherto.

[0031] The methods according to the invention, in particular the MYELO-ELISA and the MYELO-SIEFED, are particularly useful for the measurement of equine myeloperoxidase and find advantageous use in veterinary medicine, the methods being used in the diagnosis and/or the prediction of susceptibility to diseases correlated with neutrophil activation or inactivation, or being used to evaluate the immunological status of a horse.

[0032] The methods according to the invention that make use of specific antibodies, are not restricted to the measurement of equine MPO originating from neutrophils. They can easily be extended to the measurement of MPO from mammals other than horses, including humans. It should be said that antibodies specifically recognizing equine MPO do not recognize that of other species (Serteyn *et al.*, 2003, Ann. Méd. Vét. **147**:79-93). Species-specific antibodies can be raised using standard techniques if not already (commercially) available. The methods of the invention, in particular the described SIEFED method, are also not defined to MPO per se but can easily be extended to other enzymes, including but not limited to elastase, trypsin, ...

[0033] The biological sample or medium is preferably a biological fluid which can be obtained from said mammal, preferably a horse. Such biological fluid could be a cellular biological fluid or an acellular biological fluid. Said biological fluid could be venous and capillary blood serum or plasma, seminal fluid, broncho-alveolar fluid, pleural fluid, sputum, nasal fluid, ascites fluid, gastric bowel and faecal derivate samples or cerebrospinal fluid.

[0034] The biological sample or medium could also be an extract obtained from various tissues of a mammal or other complex biological samples or media which may also comprise other molecules such as proteins (albumin, lipoprotein) and reducing agents that may interfere with

adequate MPO measurement as observed for tests known in the art.

[0035] Therefore, contrary to methods of the state of the art as described for instance in WO 99/61907, the immunological detection with methods according to the invention allows to assess the natural defense capacity or ability of a mammal facing infection by measuring specifically the myeloperoxidase content originating from neutrophil cells and neutrophil cells only.

[0036] The methods according to the invention also apply to some specific diagnostic assays already proposed for the horse such as the detection of diseases of inflammatory origin, which may affect said mammal, especially the horse.

[0037] Below, some more detailed information is given on the MYELO-ELISA and the MYELO-SIEFED assays that were developed (see Figs. 1 and 2 for a general scheme). The acronym ELISA stands for Enzyme-Linked Immuno Sorbent Assay and the acronym SIEFED stands for Specific Immunological Extraction Followed by Enzymatic Detection.

[0038] The MYELO-ELISA immuno assay or method comprises the following steps. First myeloperoxidase from a biological sample taken from a mammal, preferably a horse, healthy or suspected to be diseased is immunocaptured. Immunocapture is by specific immobilized first antibodies (immobilized on a solid support such as a plastic surface of a multiwell plate). The capturing step is followed by the binding on the immobilized myeloperoxidase of another antibody (the second antibody) that is coupled to an enzymatic marker, used to reveal the reaction between the first antibodies and the myeloperoxidase. Said MPO-specific antibodies are obtained with a highly purified myeloperoxidase molecule that can be a natural or a

recombinant myeloperoxidase. Preferably a recombinant myeloperoxidase is used.

[0039] The (MYELO-)SIEFED immuno assay or method is a novel and inventive method that comprises the following steps. First there is the capture of an enzyme, for instance myeloperoxidase, obtained from a mammalian sample, preferably a horse sample. The sample may be taken from a healthy individual or one suspected to be diseased. The enzyme to measure and/or detect is captured by immobilized specific antibodies (immobilized on a solid support such as a plastic surface). The capturing of the enzyme, for instance MPO, is then followed by a washing step, whereby components that can interfere with the measurement are washed away. Enzymatic activity of said enzyme, for instance myeloperoxidase, fixed on its specific antibodies is then determined by specific techniques described hereafter. This technique does not require any extensive and laborious purification steps which would otherwise be required when working with complex samples.

[0040] Another aspect of the present invention concerns immunoassays kits or kits-of-parts comprising the elements for performing the step of these two immunoassays (MYELO-ELISA and (MYELO-)SIEFED immunoassays). Such elements may include the MPO recognizing antibodies, possibly labeled, chromogens and other substrates, buffers, diluants or washing solutions, blocking agents, ….

[0041] In an embodiment according to the present invention, the kits are kits-of-parts.

[0042] Another aspect of the invention relates to neutrophil cell activation status devices comprising one of the above-described ELISA and/or SIEFED kits.

[0043] A particular embodiment of the invention concerns a sandwich ELISA whereby the second antibody is recognized by a "revelation" antibody labelled with an

enzyme, such as an alkaline phosphatase allowing the detection of the immunological complex [the first immobilized MPO-recognizing antibody – MPO – the second MPO recognizing antibody]. By turning a substrate into a colored, phosphorescent or fluorescent reaction product, the enzyme that is linked to said second antibody allows the detection and/or quantification of the bound MPO molecule present in the sample. In a particular embodiment of the invention, the "revelation" antibody was labelled with an alkaline phosphatase and the substrate was N-nitrophenyl phosphate. Many other suitable labels and substrates are, however, known to the person skilled in the art.

[0044] Another embodiment of the invention is related to a MPO-SIEFED method whereby the MPO present in the sample is captured by immobilized specific antibodies. In this particular case, its enzymatic activity was detected via a fluorimetric reaction product of a substrate such as Amplex® Red (10-acetyl-3,7-dihydroxyphenoxazine, a "fluorogen") when said substrate is added to the bound MPO in the presence of H_2O_2. Many other detection techniques and means are available to the person skilled in the art.

[0045] It was surprisingly found that the detection sensitivity of the MPO-SIEFED method could be significantly increased by the addition of nitrites which significantly enhanced fluorescence. The preferred amount of nitrite (NO_2^-) to add to the reaction mixture is comprised between about 0.5 to about 5 ng/ml, and preferably is about 2 ng/ml. Nitrite is preferably added under the form of a salt such as a Na-salt or any other alkali or earth alkali salt (such as Li, K, Rb, Cs, Be, Mg, Ca, Sr salts) except toxic salts (such as presumably Ba, Ra or Fr salts). Amplification of the detection signal makes it possible to accurately measure and detect the enzymatic activity of an

enzyme, for instance that of MPO originating from neutrophils, in the most complex (biological) media, tissues or samples.

[0046] In a particular and preferred embodiment of the invention, the sensitivity of the enzymatic detection was increased at least 2-fold, preferably at least 5-fold, most preferably at least 10-fold or 20-fold by using nitrite as fluorescence enhancer.

[0047] This technique of fluorescence enhancement is equally well applicable to the detection of other peroxidase activities, and is applicable not only to the described SIEFED methods but to any detection method or kit that may require the use of a peroxidase enzyme.

[0048] A particular aspect of the present invention relates to the use of nitrite to enhance enzymatic detection of peroxidases. Nitrite in particular was found to increase a 10-acetyl-3,7-dihydroxyphenoxazine-induced fluorescence signal.

[0049] The interest of the above described detection techniques (ELISA AND SIEFED) is that they allow to know separately, if wanted, the total MPO concentration (active and inactive enzyme forms; by MYELO-ELISA) and the concentration of the active form only (by MYELO-SIEFED) that has been released and/or is present in biological samples, which may be complex samples.

[0050] During uncontrolled inflammatory processes, a release of active myeloperoxidase in biological fluids (e.g. blood) could be injurious for surrounding cells or tissues. SIEFED bioassays allow determination of the active part of the enzyme (potentially toxic) whereas ELISA bioassays will give information on the total concentration of the enzyme. Both tests are thus complementary.

[0051] The potential applications of ELISA and SIEFED for equine myeloperoxidase in particular are:

- the evaluation of the intensity of neutrophil activation and systemic or local inflammatory reaction, in acute or chronic inflammation pathologies (sepsis, septic shock, pulmonary inflammation pathologies, intestinal pathologies).
- the follow-up of the activation of neutrophils during therapy, taking samples of the same diseased individual or mammal at different time intervals whereby the neutrophil activation status can be followed in time.
- the early diagnostic or forecasting of some pathologies.
- the evaluation of the ability of neutrophils to destroy micro-organisms (evaluation on isolated neutrophils), a test to be applied in immunosuppression pathologies.
- the evaluation of the natural defense capacity or ability of an individual or group of individuals to fight against infections.
- the measurement of myeloperoxidase capture by other cells (in relation with their ability to fight against micro-organisms and/or to destroy them).

[0052] When examining the ability of cells other than neutrophils to fight against micro-organisms and/or to destroy them, the bioassays that have been described above are then applied to samples containing said other cell type. By comparing MPO levels obtained for said cells with neutrophil MPO levels of the healthy individuals, an estimate can be made of the capacity or ability of said cells to fight infections by micro-organisms.

[0053] The above detection techniques can further find advantageous use in the study of the efficiency of certain medicaments such as immunomodulators. MPO levels of neutrophils that have been in contact with for instance said immunomodulators are then compared with MPO levels of non-treated neutrophil cells, said MPO levels being an

indication for the neutrophil activation status and/or their ability to fight and/or destroy micro-organisms.

[0054] The detection methods according to the invention are in particular useful in the prediction, the diagnosis, possibly in a very early stage, and/or the follow-up one of the following pathologies or diseases: inflammatory diseases, digestive pathologies, strangulated intestinal pathologies, sepsis, septic shock, chronic and acute pulmonary pathologies (with invasion of the alveoli by neutrophils), ischemia-reperfusion pathologies, articular pathologies (with presence of neutrophils in the joints), colics, allergies, infections, cardiovascular diseases, …

[0055] The SIEFED detection method according to the invention is further particularly useful for the *in vitro* evaluation of the inhibitory capacity on myeloperoxidase activity of drugs (either natural products obtained from plant extracts or from animal origin, or newly synthesized molecules), allowing to distinguish between a neutralizing effect of said drugs on the products of myeloperoxidase activity (stoechiometric anti-oxidant activity) or a direct inhibitory activity on the enzyme function itself (anti-catalytic activity).

[0056] In a more general way, by comparing MPO levels measured via ELISA (active and inactive MPO measured) and SIEFED techniques (only active MPO measured), the efficiency of purification techniques can be assayed.

Short description of the figures and drawings

[0057] Fig. 1 represents a general scheme of a MYELO-ELISA.

[0058] Fig. 2 represents a general scheme of a MYELO-SIEFED with fluorescence amplification or enhancement.

[0059] Fig. 3 represents the main steps of MPO purification: as visualised with electrophoresis of pure equine MPO in non-reducing conditions [A], in reducing [A(R)] conditions, and in non-reducing conditions with enzymatic activity detection on the gel [A(O-dianisidine)]. Purification steps comprised isolation of polymorphonuclear leucocytes (PMN) from blood, extraction of PMN, dialysis, chromatography (cationic exchange, gel filtration) and electrophoresis.

[0060] Fig. 4 represents the results of an immunodiffusion test for rabbit IgG (1) and guinea pig IgG (2) obtained against equine MPO. The antibodies used in this test were polyconal antibodies (IgG) purified by affinity chromatography on Protein A Sepharose.

[0061] Fig. 5 represents MPO standard curves for a MYELO-ELISA performed with polyclonal antibodies: before (A) and after (B) logarithmic transformation (n=10). Mean OD values, standard deviations (SD) and coefficients of variation (CV) are given in the corresponding table.

[0062] Fig 6 represents MPO standard curves for a MYELO-ELISA performed with monoclonal antibodies: before (A) and after (B) logarithmic transformation (n=10). Mean OD values, standard deviations (SD) and coefficients of variation (CV) are given in the corresponding table. Curves were found to be linear for MPO concentrations ranging between 3.125 and 50 ng/ml.

[0063] Fig. 7 represents an intra-assay variation for MYELO-ELISA assays performed with polyclonal antibodies. EDTA Plasmas taken from horses with (Patho) or without (N) pathologies were diluted 40 times. Mean OD values, standard deviations (SD) and coefficients of variation (CV) are given in the corresponding table.

[0064] Fig. 8 represents an inter-assay variation for MYELO-ELISA assays performed with polyclonal

antibodies. EDTA Plasmas taken from horses with (Patho) or without (N) pathologies were diluted 40 times. Mean OD values, standard deviations (SD) and coefficients of variation (CV) are given in the corresponding table.

[0065] Fig. 9 represents the effects of sample dilution on MPO values measured via ELISA in serum and plasma (A), and on MPO values measured via ELISA in the supernatant of stimulated (PMN A) or non-stimulated (PMN NA) equine neutrophils (n=3).

[0066] Fig 10 represents the results of a MYELO-SIEFED performed with polyclonal antibodies: before (A) and after (B) linear transformation (n=3). Mean OD values, standard deviations (SD) and coefficients of variation (CV) are given in the corresponding table. Incubation time of MPO with immobilized antibodies: 2 h at 37°C. Enzymatic activity was detected with Amplex® Red as substrate.

[0067] Fig. 11 represents a MPO standard curve for a MYELO-SIEFED performed with monoclonal antibodies: before (A) and after (B) linear transformation (n=3). Mean OD values, standard deviations (SD) and coefficients of variation (CV) are given in the corresponding table.

[0068] Fig 12 represents a MPO standard curve for a MYELO-SIEFED and shows the positive effect of adding nitrites as enzymatic reaction enhancer when Amplex® Red is used as substrate (MYELO-SIEFED+).

[0069] Fig. 13 demonstrates that a MYELO-SIEFED performed with polyclonal antibodies can be efficiently used for the detection and measurement of MPO enzymatic activity in biological samples. A distinction could be made between MPO levels of non-stimulated (PMN) neutrophils and of neutrophils that were stimulated by phorbol myristate acetate (PMN + PMA). Increasing numbers of PMN were used.

[0070] Fig 14 demonstrates that a MYELO-SIEFED can be efficiently used to detect and measure MPO enzymatic

activity in different biological samples such as plasma and seminal liquid, and that a distinction can be made between normal (healthy) and pathological samples. SD: standard deviation; CV: coefficient of variation; N: normal samples; P: pathological samples.

Detailed description of the invention

[0071] The present invention will now be described in detail in the following description of preferred embodiments of the present invention in reference to the enclosed figures.

[0072] The examples given below are offered by way of illustration, not by way of limitation.

Examples

Example 1: Purification of equine myeloperoxidase (MPO)

[0073] MPO was extracted from equine polymorphonuclear leucocytes (PMN) isolated from whole blood by sedimentation on a Ficoll-Paque density gradient. The purification was performed, with some modifications, following a previously described technique (Mathy-Hartert et al. 1998, Can J Vet Res. 62:127-32). Briefly, packed neutrophils were homogenized in sodium acetate buffer (0.2 M Na acetate; 1 M NaCl; pH 4.7) containing 1 % cetyltrimethylammonium bromide (CETAB). The supernatant was collected by centrifugation and dialysed. Dialysis allowed precipitation of elastase and cathepsin G, while MPO was recovered in the supernatant. MPO was furhter purified by two chromatographic steps: ion exchange (Hiload SP Sepharose) with a NaCl gradient followed by gel filtration chromatography on Hiload Superdex 200 (elution with a NaCl-acetate buffer). The purity of MPO was assessed by enzymatic assays (orthodianisine technique) and by

electrophoresis on polyacrylamide gels (ExcelGel SDS, gradient 8-18) (Fig. 3).

Example 2: Preparation and purification of MPO antibodies

[0074] For the production of polyclonal antibodies, antisera were raised in rabbits and guinea pig by intradermic injection of 100 μg of pure equine MPO. Booster injections were given at 15 days intervals with 50 μg of MPO. Blood samples were collected 10 days after each booster injection. After the last booster, the two animals were ex-sanguinated. Purification of the polyclonal antibodies (immunoglobulin, or IgG) from antisera was realized by affinity chromatography on a Protein A Sepharose column. Reactivity of the two antibodies against equine MPO was tested qualitatively by immunodiffusion (Ouchterlony technique) (Figure 4).

[0075] Monoclonal antibodies and corresponding hybridoma have been obtained and tested for their reactivity against equine MPO. Among several convenient hybridoma, two were selected for the production of monoclonal antibodies to be used in the ELISA and SIEFED techniques.

Example 3: Sandwich ELISA technique to measure total (active and inactive) neutrophil MPO content

[0076] For the measuring of the activation status of neutrophils by measuring both active and inactive MPO (1), a classical "Sandwich" Elisa method was designed (figure 1). The ELISA developed for MPO (1) measurement is further referred to as a MYELO-ELISA. Rabbit IgG, the primary antibody (2), is immobilized (coated) in excess (3 μg/ml) onto microtitration wells (3) (Cliniplate EB, Labsystem). Standard or test antigen (equine MPO (1)) is incubated

overnight with the primary antibody (2) at 4°C. After washing (0.9 % NaCl solution containing 0.1 % tween 20), the immobilized antibody-antigen complex is incubated (2 h, 37°C) with an excess (5 µg/ml) of guinea pig IgG, the secondary antibody (4). After washing, a third antibody (5) produced against guinea pig IgG is added. This third IgG (5) (goat IgG) is labelled with alkaline phosphatase (6) and recognizes the "sandwich" complex "primary antibody-MPO-secondary antibody". After washing, phosphatase activity is detected by incubation (30 min, 37°C, in the darkness) with a paranitrophenyl phosphate solution (phosphatase substrate, Sigma). The reaction is stopped with NaOH and the absorbance (405 nm) is measured with a Multiscan Ascent apparatus (Labsystem) (7). All the volumes added into the wells comprise 100 µl, except for washing (300 µl) and for the substrate solution (200 µl). Controls (blank) and dilutions of the standard MPO and samples were established with dilution buffer [PBS (20 mM phosphate, 137 mM NaCl and 2.7 mM KCl pH 7.4) to which 5g/L bovine serum albumin and 0.1 % tween 20 was added]. The same ELISA technique was developed also for monoclonal antibodies as primary antibody.

[0077] The absorbance response obtained with such ELISA assays is directly proportional to the quantity of sandwich complex formed, in other words to the concentration of MPO in the sample.

<u>Example 4: SIEFED technique to measure active neutrophil MPO content</u>

[0078] SIEFED ("specific immunological extraction followed by enzymatic detection") is an immunodetection technique consisting of two steps:

- the capture of an enzyme such as (equine) MPO (1) from biological samples by immobilized specific antibodies (2), followed by

- the enzymatic detection of the enzyme such as MPO (1) immobilized on the antibodies that are coated onto a solid support (3)(figure 2).

[0079] Contrary to the above described ELISA test, the SIEFED techniques measures active MPO only. In a way, both tests are thus complementary.

[0080] As for the ELISA test, the primary antibody, that captures MPO, is rabbit IgG (3µg/ml). Standard MPO or unknown sample is incubated 2 h at 37°C. After washing, the peroxidase activity of MPO is detected by adding 100 µl of a 10 µM H_2O_2 solution and 40 µM of Amplex® Red (10-acetyl-3,7-dihydroxyphenoxazine; Molecular Probes) in phosphate buffer (50 mM, pH 7.5). After incubation in the darkness (30 min, 37°C), the fluorescence is read at 590 nm with a Fluoroskan Ascent apparatus (Labsystems). The volumes of the primary antibody and the sample put in the wells, are 200 µl. Controls (blank) and dilutions of the samples are established with dilution buffer.

[0081] The same technique was developed for monoclonal antibodies that recognize the active form of MPO.

[0082] An original technique of enhancement of the peroxidase response of MPO has been developed, leading to an increased fluorescence response and to an increase of the sensibility of the MYELO-SIEFED. Enhancement of fluorescence was surprisingly obtained when adding a defined concentration of nitrites ions (about 10 mM) to the Amplex® Red solution. This sensibility enhancement technique is applicable to the enzymatic detection of other

peroxidases as well in other medical or industrial detection processes.

Results and discussion

Purified MPO retained its enzymatic activity

[0083] Electrophoresis of purified equine MPO shows 3 bands: two at molecular weight near 120 kDa (native enzyme) and one at 96 kDa (precursor) (Fig. 3). When MPO is treated with dithiothreitol (prior to loading onto the gel in order to break internal disulphide bridges and to release the subunits structure of the enzyme), the band at 96 kDa remains, the bands at 120 kDa disappear and two bands appear at 64 kDa and 16 kDa corresponding respectively to the heavy and light subunits of the enzyme. A weakly stained band also appears at a molecular weight of 40 kDa, that can result from an intramolecular disulphide bridge breaking or that represents the heavy subunit without the prosthetic group. Another weak band appears at 76 kDa, that could be attributed to the hemi-enzyme (heavy and light subunits). The peroxidase activity (defined as the stain of the protein bands on the gel by orthodianisidine in the presence of H_2O_2) showed activity at the 120 kDa bands under non reducing conditions.

Raised polyclonal and monoclonal antibodies efficiently recognize MPO

[0084] A good reactivity (presence of precipitation arcs) was observed between equine MPO and IgG from rabbit and guinea pig (Fig. 4, Ouchterlony detection technique). Similar results are obtained with several monoclonal antibodies, two of which were selected for further ELISA and SIEFED development.

MPO standard curve for the developed ELISA test

[0085] An MPO standard curve was obtained by plotting the absorbance values at 405 nm as a function of standard MPO concentrations measured via the developed ELISA test. This standard curve is a classical one, reaching a plateau for the highest MPO concentrations. An almost linear curve is obtained when MPO concentrations are expressed in the logarithmic form (Fig. 5). The table shown in Fig. 5 lists the absorbance values (405 nm), standard deviation (SD) and intra-assay variation coefficient (CV in %) obtained for an equine MPO 2-fold dilution series ranging from 0,78 to 100 ng/ml MPO in the dilution buffer (for the composition: see Example 3).

[0086] Standard curves obtained with a monoclonal antibody are shown in Fig. 6. The results are similar to those obtained with the polyclonal antibodies (Fig. 5).

[0087] MPO standard curves allow quantification of the amount of MPO detected. Monitoring of disease progression benefits from such quantification. By comparing mean MPO levels of healthy with diseased individuals, cut-off values can be established that allow distinction between healthy and diseased mammals. Preferably such cut-off values are established for the different biological samples assayed for neutrophil MPO levels.

Developed MPO ELISA test allows detection of equine MPO in acellular complex samples such as plasma

[0088] MPO levels were detected in biological samples consisting of plasma drawn from blood with different anticoagulants (EDTA, citrate, heparin), serum (Fig. 9A) or supernatant isolated from stimulated or unstimulated neutrophils (PMN) (Fig. 9B).

[0089] It was found that the best sampling technique for MPO measurement in plasma (as true witness of *in vivo*

neutrophil degranulation) is to collect blood onto EDTA, which allows one to get a plasma value of MPO that is stable with time. The plasma drawn onto heparin and the serum allow *in vitro* degranulation of neutrophils, leading to artefactual values of MPO.

[0090] An important liberation of MPO was observed in the supernatant of excited neutophils in comparison to non-excited ones. Intra- (Fig. 7) and inter- (Fig. 8) assay coefficients of variation (witness of the precision of the technique) were established for plasma taken from healthy horses and horses with inflammation pathologies.

[0091] The highest concentrations of MPO were observed in plasma from horses with intestinal strangulated pathologies.

[0092] The above demonstrates that the tests of the present invention are sensitive, accurate and clearly able to make a distinction between healthy and pathological animals. They further demonstrate that the measurement of plasma MPO can be taken as the witness of neutrophil activation and are positively correlated to certain pathologies.

[0093] The test was also applied with success to peritoneal fluids and seminal liquids.

<u>Sensitivity and precision of the developed ELISA test</u>

[0094] For the developed ELISA test for equine MPO (MYELO-ELISA), the sensitivity of the assay is about 2 ng/ml. Good intra- and inter-assay precisions are obtained for standard curves (inferior to 8 %) and biological samples (generally inferior to 10 %). The mean MPO value measured in normal horses was 181.8 ± 64.7 ng/ml in EDTA-anticoagulated plasma (n=38).

SIEFED technique to measure active MPO levels in tissue extracts (MYELO-SIEFED) and to distinguish the active MPO form from the total MPO (inactive and active) form in biological samples

[0095] The enzymatic activity of MPO produces HOCl (hypochlorous acid) or NaOCl (sodium hypochlorite) and other oxidant species potentially toxic if the enzyme acts directly in contact with tissues or into the cells, thus in places other than in the phagolysosome. MPO can be present in biological fluids in an inactive form (inhibition by oxidation or by specific inhibitors). It is interesting to distinguish the active MPO from its inactive form in biological samples.

[0096] A direct enzymatic measurement of MPO in biological fluids is impossible by the presence of proteins, mainly albumin. Before measurement in complex biological medium, the enzyme would have to be extracted by long purification procedures implicating chromatography separation. The originality of the SIEFED technique lies in the fact that active MPO can be detected by performing two easy steps only, which are the capture of equine MPO from the biological sample by specific immobilized antibodies, followed, after washing (elimination of albumin and other proteins) by a direct detection of the enzymatic activity with an appropriate substrate (mainly high sensitivity).

[0097] Indirectly, the SIEFED technique will indicate any anomalies that might have arisen during MPO isolation and purification.

MPO standard curve for the SIEFED test

[0098] A MPO standard curve was obtained by plotting the fluorescence values (corresponding to MPO activity), read at 590 nm, as a function of the standard MPO concentrations measured with the developed SIEFED test.

[0099] A standard curve obtained with increasing concentrations of MPO is shown in Fig. 10A. An almost linear curve is obtained with the mathematical transformation of the fluorescence values (Fig. 10B). The corresponding table of Fig. 10 lists the mean absorbance values, standard deviation, and intra-assay coefficient variation (CV (%)) as an indication of the assay precision) obtained for the measured MPO concentrations (2-fold dilution series). Reaction time with Amplex Red was 30 min. Incubation time of MPO with immobilized polyclonal antibody was 2 h at 37°C.

[0100] A similar standard curve was obtained when monoclonal antibodies were used (Fig. 11).

[0101] The addition of nitrite ions (about 10 mM) under the form of a salt (sodium salt) to the reaction medium could enhance until tenfold the sensibility of the SIEFED assay (Fig. 12).

Developed MYELO-SIEFED test allowed to detect active equine MPO in acellular complex samples such as plasma

[0102] MPO levels were measured via the developed SIEFED test in biological samples consisting of plasma, serum, seminal liquid (Fig. 12), and supernatant isolated from excited or not excited neutrophils (PMN) (Fig. 13).

[0103] MPO levels can be measured via SIEFED in biological samples, diluted or undiluted. Concentrated samples often have to be diluted to avoid interference of proteins (abundantly) present in the biological medium, especially albumin. The addition of an enhancer (nitrites) of the peroxidase enzymatic activity allows using a fivefold sample dilution.

Sensitivity and precision of the developed SIEFED assay

[0104] The sensitivity of the SIEFED assay for active equine MPO, developed with polyclonal or monoclonal antibodies and addition of the nitrite enhancer was about 2 ng/ml. Good intra-assay precision was obtained for standard curves and for biological samples (inferior to 10 %).

CLAIMS

1. An *in vitro* method for measuring the activation status of neutrophil cells in a biological sample obtained from a mammal, preferably a horse, which method specifically measures the myeloperoxidase (MPO) content only, said content being correlated with said cell activation status, said method comprising the steps of:

- obtaining a biological sample, preferably a biological fluid from said mammal, preferably a horse, said sample containing said neutrophil cells and/or MPO released by said cells,
- capturing MPO present in said biological sample via an MPO-specific polyclonal or monoclonal antibody,
- detecting and/or measuring either total (active and inactive) MPO or exclusively active MPO present in said biological sample,
- possibly comparing the measured MPO values with normal MPO levels obtained from a significant number of healthy mammals, preferably horses,
- optionally quantifying MPO levels using a standard MPO curve, and
- relating the normalized MPO levels measured to an activity status of said cells indicative of the presence, absence or condition of a disease or immunological status.

2. The method according to claim 1 which is a sandwich ELISA detection method, wherein detection is through a second enzymatically labelled antibody binding to a first immunological complex formed by a first MPO-recognizing immobilized antibody and MPO, the method developed to detect and/or measure MPO, preferably equine MPO.

3. The method according to claim 1 which is a SIEFED detection method, wherein the step of

immunocapturing MPO is followed by a washing step to remove any components that can interfere with the measurement of MPO enzymatic activity, the enzymatic activity of said myeloperoxidase fixed on its specific antibodies then detected by adding a specific substrate to be transformed by the MPO into a visible, preferably a fluorescent reaction product.

4. The method according to claim 3 wherein H_2O_2 and a suitable chromogen such as 10-acetyl-3,7-dihydroxyphenoxazine are added to the reaction medium, and preferably also a sufficient amount of nitrite to enhance a myeoloperoxidase enzymatic reaction.

5. The method according to any of claims 1 to 4 wherein said biological sample is a cellular or acellular sample selected from the group consisting of arterial, venous and capillary blood, serum, plasma, seminal fluid, broncho-alveolar fluid, urine, saliva, endotracheal fluid, peritoneal fluid, uterine irrigation liquids, sputum, broncho-alveolar fluid, nasal fluid, gastric bowel and faecal derivate samples, cerebrospinal fluid and tissue extracts.

6. The method according to any of claims 1 to 5 wherein a neutrophil cell activation status is measured and correlated with a disease and/or pathology.

7. An ELISA kit for measuring the activation status of neutrophil cells in a biological sample obtained from a mammal, preferably a horse, which ELISA kit specifically measures the total (active and inactive) myeloperoxidase (MPO) content only, said content being correlated with said cell activation status, said ELISA kit comprising the necessary elements for:

- immunocapturing MPO (1) that is present in a biological sample, preferably a biological fluid, obtained from a mammal, preferably a horse, and containing said

neutrophil cells or MPO released by said cells, said immunocapturing being preferably obtained by a first MPO-recognizing polyclonal or monoclonal antibody (2) immobilized to a solid support (3),

- detecting and/or measuring active and inactive MPO present in said biological sample, preferably by a second enzymatically labelled MPO-recognizing polyclonal or monoclonal antibody (4) for detection of immunocaptured MPO (1),

- comparing the measured MPO values with normal MPO levels obtained from a significant number of healthy mammals,

- optionally quantifying MPO levels using a standard MPO curve, and

- relating the normalized MPO levels measured to an activity status of said cells indicative of the presence, absence or condition of a disease or immunological status,

said detection and/or measurements specifically and accurately representing MPO levels in any type of biological sample.

8. A SIEFED kit for measuring the activation status of neutrophil cells in a biological sample obtained from a mammal, preferably a horse, which SIEFED kit specifically measures the active myeloperoxidase (MPO) content only, said content being correlated with said cell activation status, said SIEFED kit comprising the necessary elements for:

- immunocapturing MPO (1) present in a biological sample, preferably a biological fluid, obtained from a mammal, preferably a horse, the sample containing said neutrophil cells or MPO released by said cells,

- detecting and/or measuring active MPO present in said biological sample, whereby nitrite may be added to the

reaction medium to amplify a myeoloperoxidase enzymatic reaction,

- comparing the measured MPO values with normal MPO levels obtained from a significant number of healthy mammals, preferably horses,

- optionally quantifying MPO levels using a standard MPO curve, and

- relating the normalized MPO levels measured to an activity status of said cells indicative of the presence, absence or condition of a disease or immunological status,

said detection and/or measurements specifically and accurately representing active MPO levels in any type of biological sample.

9. The use of a method according to any of claims 1 to 6 or of a kit according to any of claims 7 to 8

- for the detection and/or prediction of a disease or pathology, preferably one selected from the group consisting of chronic or acute inflammatory diseases, digestive pathologies, strangulated intestinal pathologies, sepsis, septic shock, chronic and acute pulmonary pathologies (with invasion of the alveoli by neutrophils), ischemia-reperfusion pathologies, articular pathologies (with presence of neutrophils in the joints), colics, allergies, infections and cardiovascular diseases,

- to follow-up neutrophil cell activation during therapy of a diseased mammal, preferabley a horse,

- to evaluate the (natural) ability of neutrophil cells to fight against micro-organisms and/or to destroy them,

- to evaluate the efficiency of immunomodulators or the *in vitro* inhibitory capacity of drugs by comparing the neutrophil activation status of treated and non-treated neutrophils,

- to evaluate the ability of neutrophils treated with said modulators and/or drugs to against micro-organisms and/or to destroy them, or

- to evaluate the natural defense capacity or ability of a mammal, preferably a horse, to fight against micro-organisms.

10. The use of nitrite, preferably a Na-salt thereof, to enhance enzymatic detection of a peroxidase, preferably to enhance a 10-acetyl-3,7-dihydroxyphenoxazine-induced fluorescence signal.

11. The use of both an ELISA and a SIEFED method to distinguish between total and active MPO content.

ABSTRACT

METHOD AND KIT FOR THE MEASUREMENT OF NEUTROPHIL CELL ACTIVATION

The present invention is related to accurate detection methods for the measurement only of myeloperoxidase (MPO) levels of neutrophils, preferably equine neutrophils, in complex biological samples. The present invention is further related to ELISA and SIEFED assays for such detection. SIEFED detection sensitivity of active peroxidase activity was found to be enhanced by the addition of nitrite.

Such MPO measurement finds its use in many applications such as the prediction, diagnosis and/or monitoring of pathologies correlated with neutrophil activation and/or destruction; the evaluation of drugs and/or immuomodulators; the assessment of immune responses, either natural and/or after treatment with immunomodulators and/or drugs; and the study of cells and their ability to fight microorganisms and/or to destroy them.

(Fig. 2)

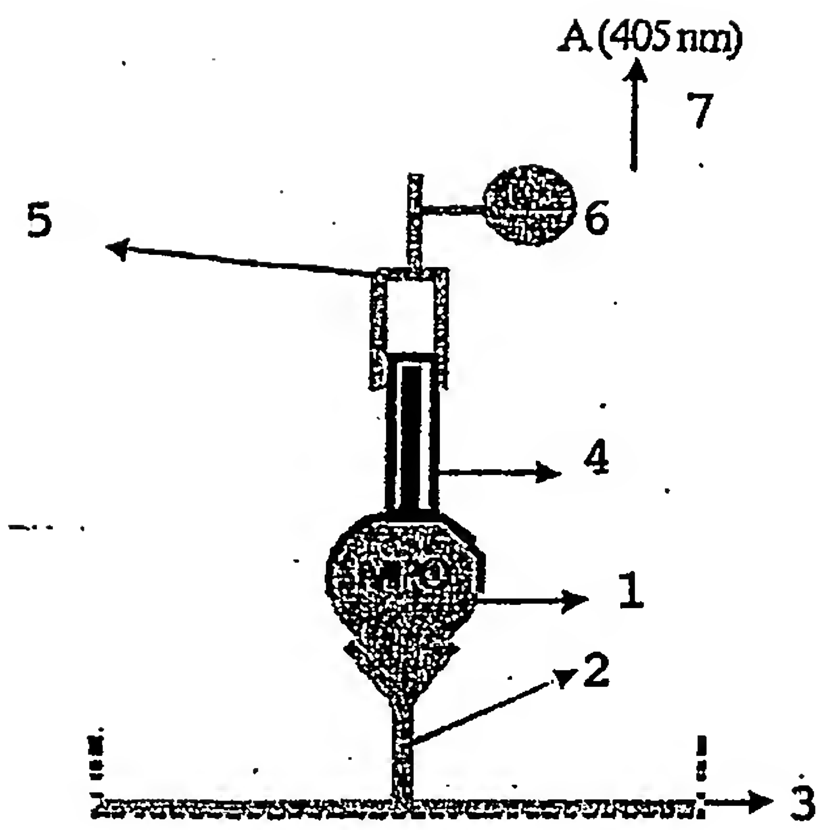

A (405 nm)
7
5
6
4
1
2
3

Fig. 1

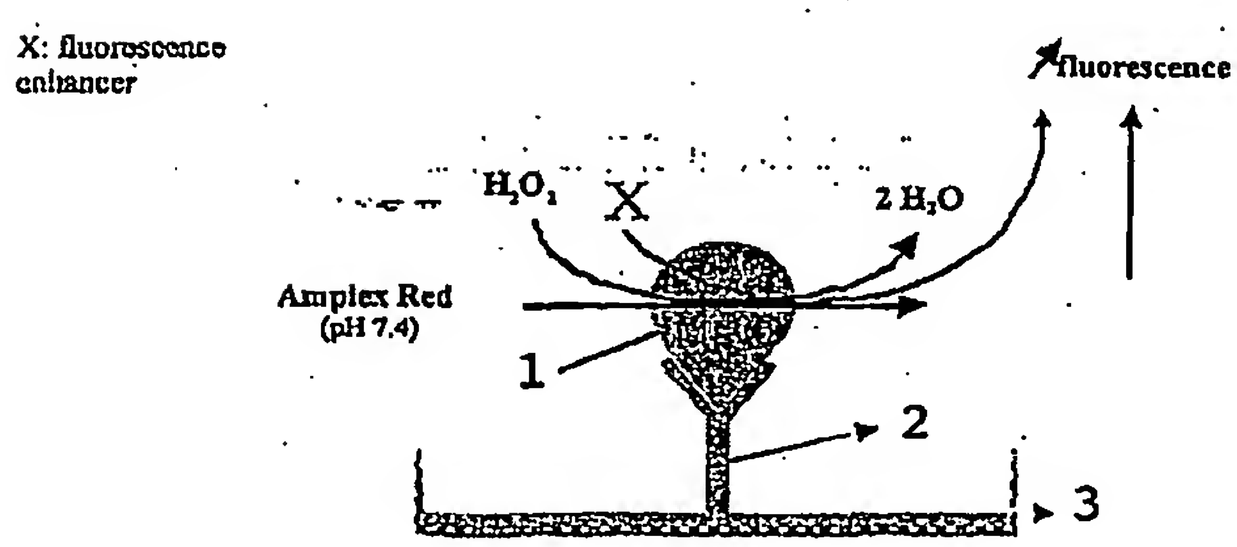

X: fluorescence enhancer
fluorescence
H_2O_2
X
2 H_2O
Amplex Red (pH 7.4)
1
2
3

Fig. 2

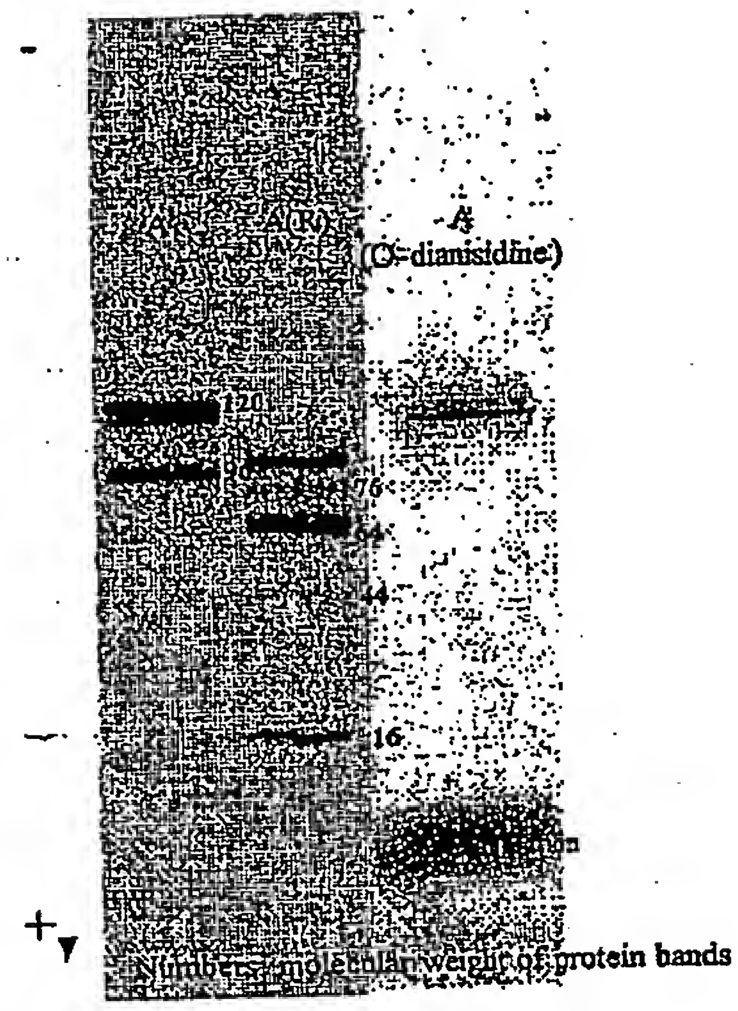

-
(O-dianisidine)
120
76
16
+
Numbers = molecular weight of protein bands

Fig. 3

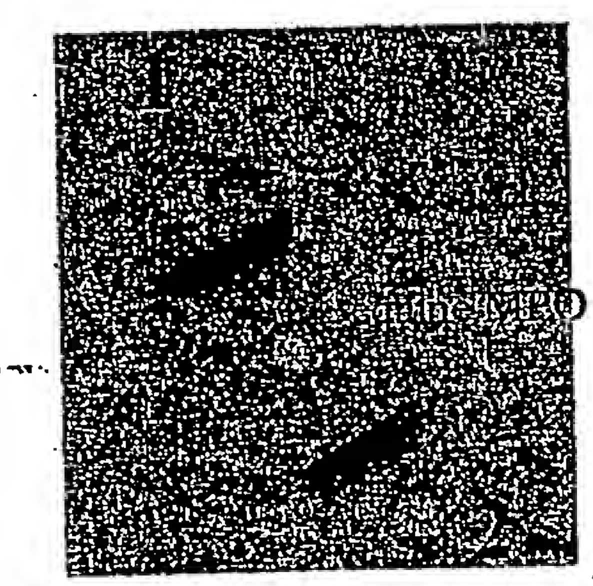
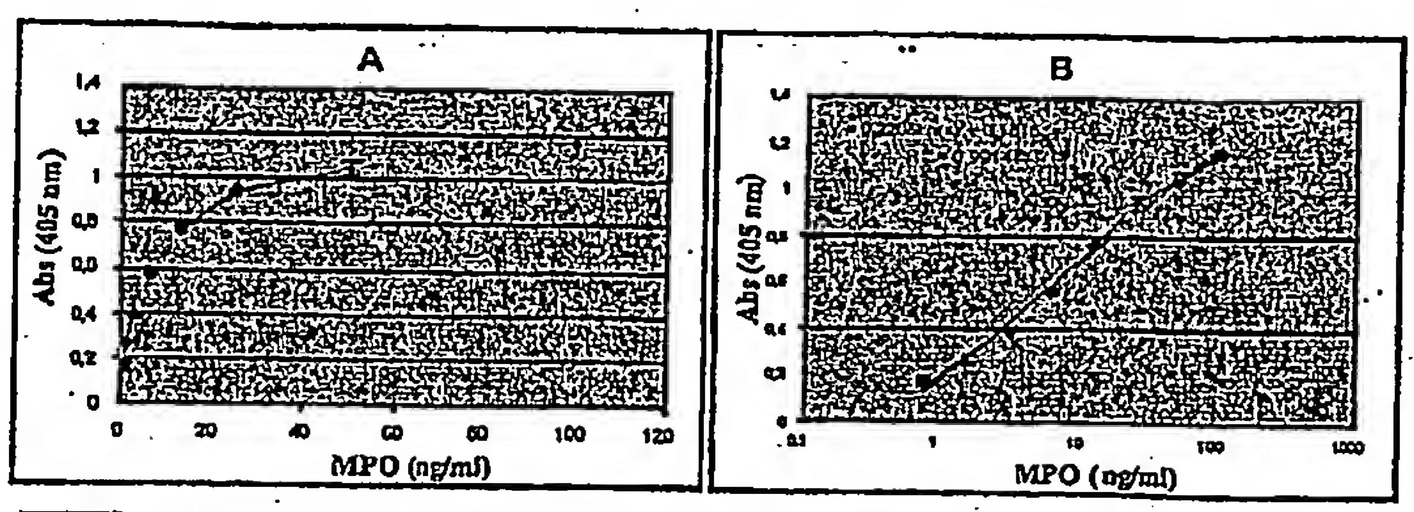


Fig. 4



MPO (ng/ml)	Mean (n = 10)	SD	CV (%)
0,78	0,1629	0,0109	6,7
1,56	0,2621	0,02	7,6
3,125	0,3871	0,0139	3,6
6,25	0,5616	0,0146	2,6
12,5	0,7623	0,0125	1,6
25	0,9444	0,0274	2,9
50	1,0528	0,0324	3,1
100	1,1678	0,0335	2,9

Fig. 5

MPO ng/ml	Mean n= 10	SD	CV %
0,78	0,058	0,01	17,24
1,56	0,114	0,009	7,89
3,125	0,263	0,008	3,04
6,25	0,418	0,023	5,50
12,5	0,702	0,032	4,56
25	0,976	0,019	1,93
50	1,264	0,064	5,06

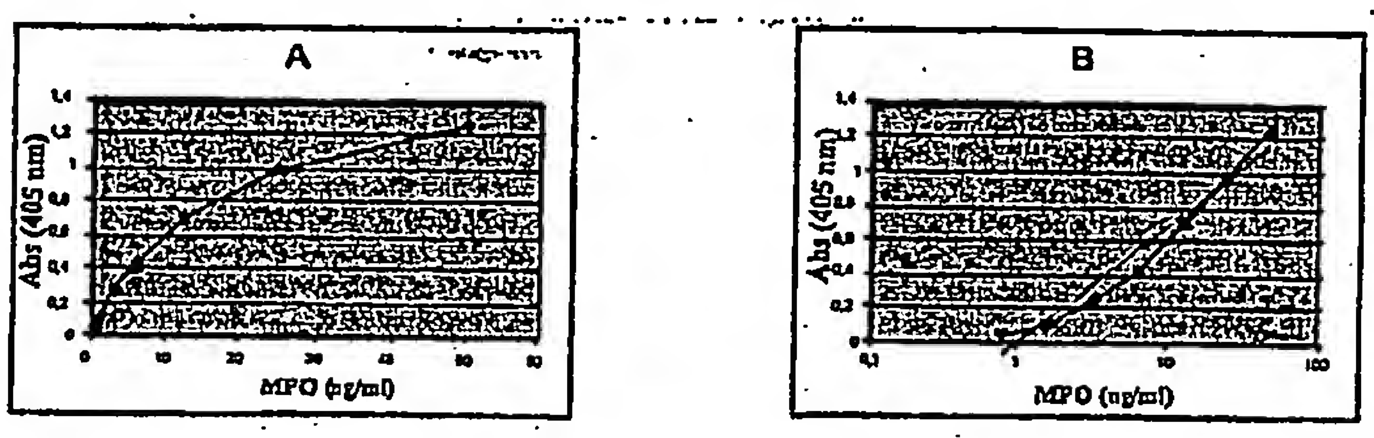
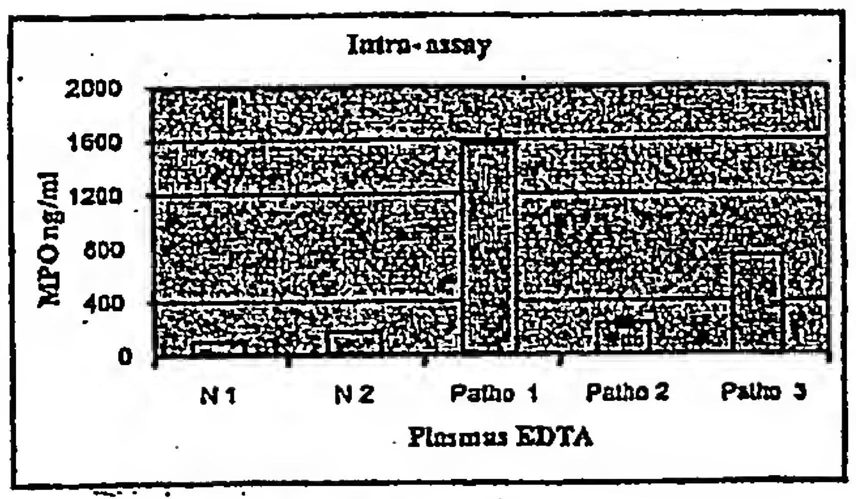
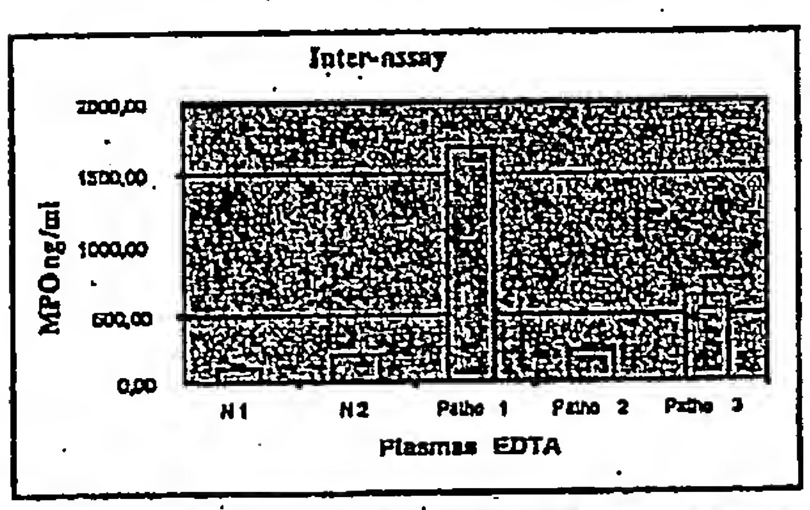


Fig. 6



Plasmas	Mean (n=3)	SD	CV (%)
N 1	114,08	3,55	3,11
N 2	177,18	7,14	4,03
Patho 1	1570,47	87,78	5,59
Patho 2	251,48	28,31	11,28
Patho 3	748,67	16,82	2,25

Fig. 7



Assay numbe	N1	N2	Patho 1	Patho 2	Patho 3
1	114,06	177,16	1670,47	251,48	748,67
2	124,42	205,14	1763,12	230,21	658,53
3	126,91	219,88	1703,56	264,28	673,61
Mean	121,60	200,72	1679,06	248,65	626,94
SD	6,81	21,70	98,64	17,20	105,68
C.V.	5,60	10,81	5,87	6,92	16,86

Fig. 8

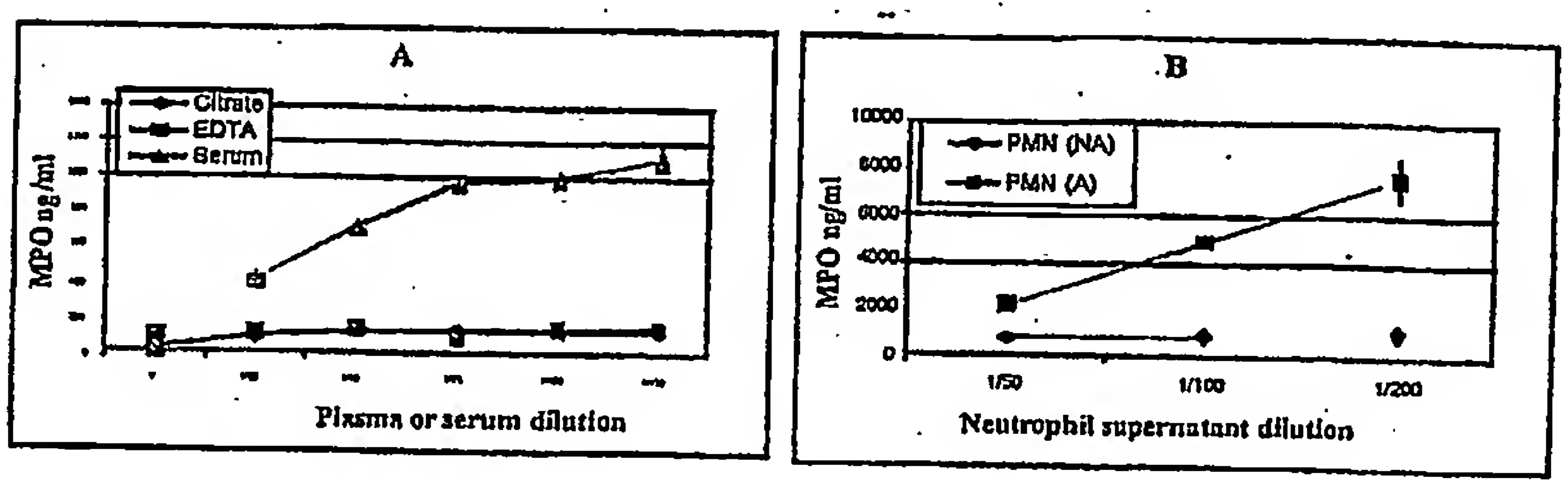


Fig. 9

MPO (ng/ml)	Mean	SD (n=3)	CV (%)
18,00	24,826	0,4850	1,95
12,00	10,585	0,2801	2,65
8,00	4,428	0,4323	9,76
5,30	1,250	0,0683	5,46
3,65	0,392	0,0213	5,43
2,37	0,144	0,0026	1,78
1,58	0,095	0,0041	4,34
1,05	0,060	0,0030	4,89

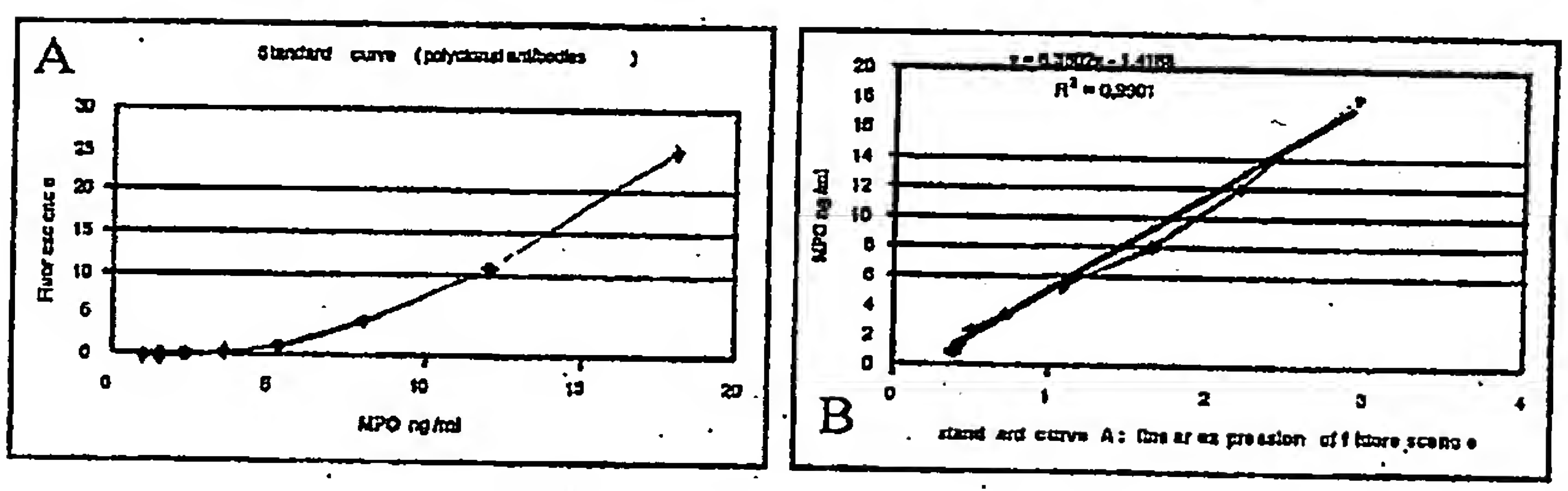


Fig. 10

MPO (ng/ml)	Mean	SD (n=3)	CV (%)
18	14,062	0,3966	2,8202
12	5,077	0,2441	4,8074
8	1,379	0,0708	5,1363
5,3	0,517	0,0168	3,2399
3,55	0,165	0,0103	6,2617
2,37	0,059	0,0062	10,490 5
1,58	0,053	0,0099	18,602 0
1,05	0,015	0,0047	31,315 7

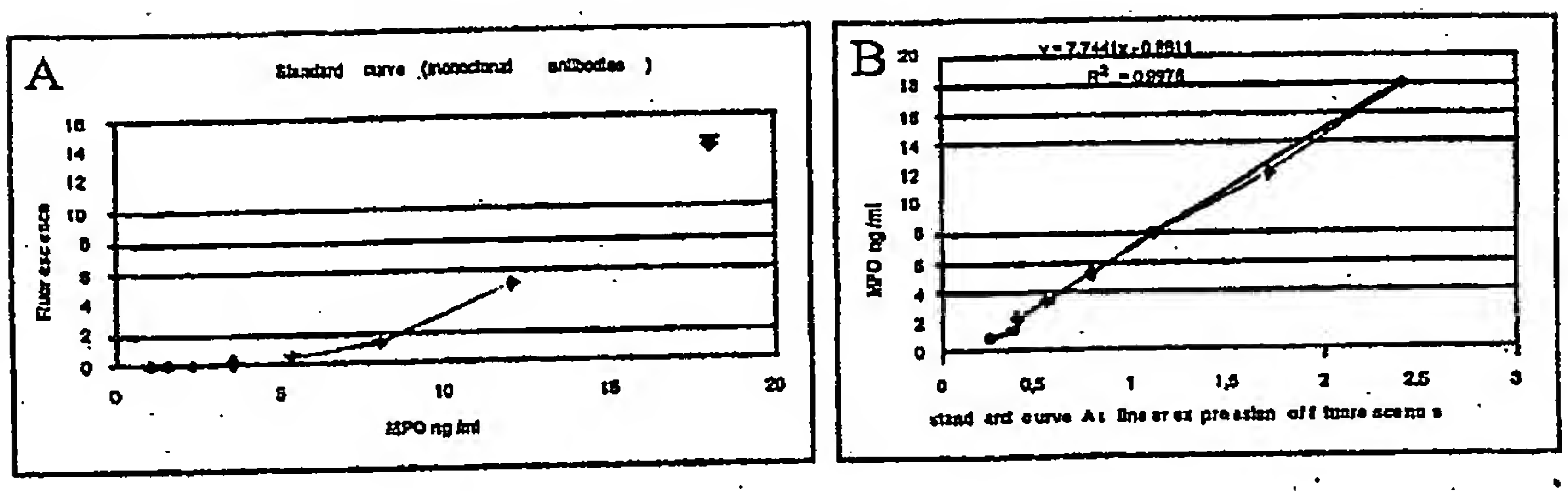


Fig. 11

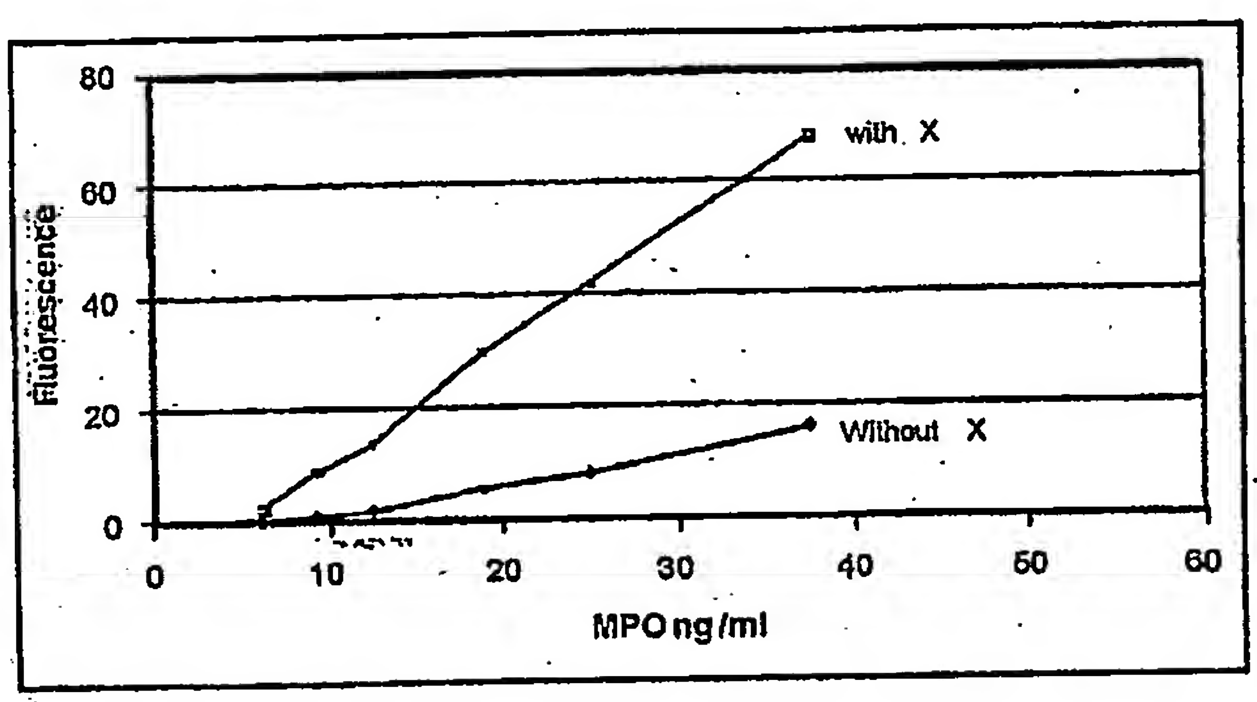


Fig. 12

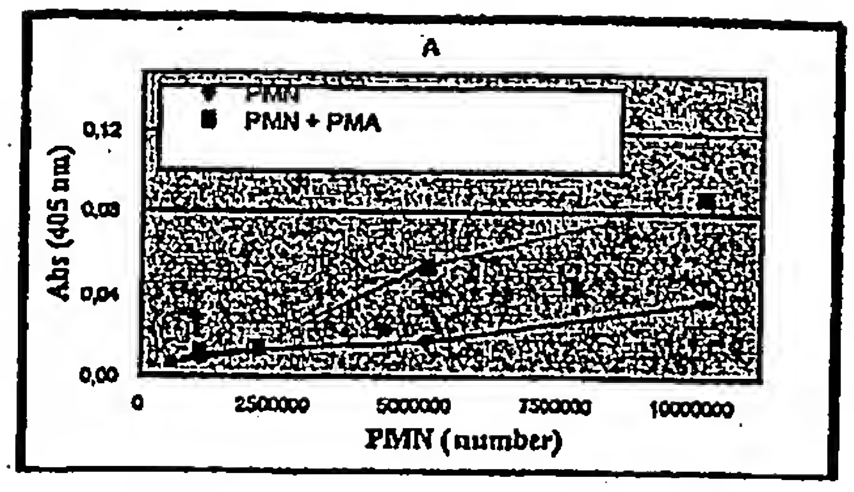


Fig. 13

	Active MPO concentration (ng/ml)		
		Undiluted samples	
	Mean	SD	CV (%)
Plasma N			
n=3	1,200	0,083	6,90
n=4	1,604	0,054	3,45
n=4	1,555	0,076	4,89
n=3	1,615	0,019	1,20
n=3	2,153	0,081	3,29
Plasma P			
n=3	3,073	0,353	11,55
n=3	1,442	0,042	2,91
n=3	17,792	1,506	8,45
Seminal liq. N			
n=3	5,273	0,19	3,60
n=3	2,483	0,224	0,02
n=3	1,652	0,023	1,43
Seminal liq. P			
n=3	6,598	0,633	8,62
n=3	1,397	0,062	4,44
n=3	1,6	0,016	1,00

Fig. 14